

# Cell Lysis and Immunoprecipitation Protocol with Triton Buffer

Part No. TIB-1, 1X, 60 ml, pH 7.4

Safety: Contains irritants. Avoid ingestion, skin and eye contact.

## Background

The FIVEphoton Biochemicals Triton Immunoprecipitation Buffer (TIB-1) is formulated to extract transmembrane and soluble cytosolic proteins from cells, yet also maintain charge-charge and hydrogen bond interactions. The buffer has its broad applicability as a standard cell lysis buffer as well in “pull down - immunoprecipitation” procedures.

Following cell lysis and solubilization by the Triton buffer, an immunoprecipitating antibody is added to bind to one of the assembled protein species in the pair. A solid support, such as Protein-G Sepharose, is added to link the antibody-assembled protein complex. Centrifugation is then used to “pull-down” the protein-antibody-solid support. Following washes of unbound proteins, the assembled protein-antibody complex is eluted and analyzed.

## Required Equipment and Reagents:

1. Microcentrifuge at 4°C
2. Rocker Table
3. Cold room or refrigerated case.
4. Antibody suitable for immunoprecipitation, as specified by the vendor. Generally, this antibody binds to the native protein conformation.
5. Immunoprecipitation beads to provide the solid support, such as Protein A or G Sepharose.

## Suggested Controls and Samples for Comparisons:

1. Immunoprecipitation steps with an irrelevant antibody to control for appearance of spurious protein bands.
2. Resolution of total cell lysate, the unbound fraction and the immunoprecipitated materials in Western blots to determine whether protein fractionation and enrichment occurred.
3. Western blot to detect the protein (i.e. antigen) directly bound to the immunoprecipitating antibody. This control verifies that immunoprecipitation occurred, but does not provide evidence of a complex between/among two or more proteins.

## Protocol:

### A. Cell Culture:

It is recommended that cells are cultured to 80-90% confluency prior to performing cell lysis and immunoprecipitation. The cells should be washed free of serum proteins using PBS prior to performing immunoprecipitation to diminish the appearance of contaminating serum protein bands in downstream Western blots.

### B. Cell Lysis:

We recommend using 300 µl of Triton Buffer for one to three 10 cm cell culture dishes of cells. Scale accordingly for other numbers or sizes of cell culture dishes. Prior to lysis, make the Triton Buffer ice cold and add protease and phosphatase inhibitors. (FIVEphoton Biochemicals markets a protease inhibitor cocktail (Part No. PI-1) and a Phosphatase Inhibitor Cocktail (Part No. PIC-1) that can be added to the cell lysis solutions). All procedures should be performed with cold Triton Buffer.

1. Apply the ice cold Triton Buffer solution to cells (300 µl of Triton buffer for one to three 10 cm plates of cells. Scale accordingly for other numbers of plates or sizes of plates) for 30 minutes: Place the Triton Buffer solution with cells in a 1.5 ml microcentrifuge tube in a ice bucket, while occasionally briskly tapping the tube to facilitate cell membrane dissolution. You can also use a rocker table to rotate the solution of cells during this period to further facilitate cell membrane dissolution and protein extraction. With successful cell lysis,

you should observe insoluble threads of DNA in the solution. Briskly tapping the tube facilitates the dissolution of membranes and release of the DNA threads.

2. Centrifuge the cell lysate in a refrigerated microcentrifuge for 15 min at full speed. Collect the supernatant fraction, which contains extracted membrane and cytosolic proteins. Dispense this supernatant into another 1.5 ml microcentrifuge tube that is placed in ice. This supernatant will be used for immunoprecipitation. Collect a small aliquot of this **unfractionated** supernatant for Western blot comparisons to the immunoprecipitated-fractionated materials. The unfractionated supernatant can also be stored at -20°C or -80°C for longer term storage. Immunoprecipitation can be performed at a later time.

*C. Immunoprecipitation:*

1. Add immunoprecipitating antibody to the lysate using the antibody titer recommended by the antibody manufacturer (or determine empirically the optimal antibody titer for immunoprecipitation). Place the tube with lysate and antibody on a rocker table in a refrigerated area (such as a cold room) for 1 hr to overnight.
2. Add approximately 60 µl of immunoprecipitation beads (e.g. Sepharose-G beads) for 300 µl to 2 ml of cell lysate supernatant. Place the tube on the rocker table for another 1 hr in a refrigerated area to generate the antigen-antibody-solid support complex.
3. Using a microcentrifuge, or a table top minicentrifuge, wash the immunoprecipitating beads of unbound proteins, by first sedimenting the beads at 3000 rpm for 5 minutes. Remove the first supernatant without disturbing the immunoprecipitating beads, and store this supernatant at -20°C or -80°C; the **unbound fraction** is this supernatant.

Add an additional aliquot of 300 µl of ice cold Triton buffer with protease and phosphatase inhibitors to the immunoprecipitation beads, gently rotate the tube 180° by hand three times and centrifuge again at 3000 rpm for 5 minutes. Remove the supernatant, which can be discarded. Repeat two more washes of the immunoprecipitating beads in the same manner. After the last wash, use a microcentrifuge to sediment the beads at 14000 rpm for 15 min. Remove as much of the wash solution as possible using a pointed plastic Pasteur pipette.

*D. Elution of immunoprecipitated sample and Western Blot:*

1. There are several methods to elute the antibody-assembled protein complex from the immunoprecipitating beads. The simplest for subsequent Western blotting is apply Laemmli Sample Buffer (LSB; approximately 100 µl LSB for 60 µl of immunoprecipitation beads), vortex at full speed for 30 sec, and then heat at 60°C for 10 min. This supernatant with Laemmli Sample Buffer contains the eluted proteins that can be resolved in Western blots. In subsequent gels and Western blots, in consecutive lanes resolve the unfractionated supernatant, the unbound fraction and the eluted immunoprecipitated sample.

**Troubleshooting:**

1. Immunoprecipitation did not occur. Resolution: 1) You may have to empirically identify an antibody for immunoprecipitation that recognizes the native conformation of the epitope. 2) The protein complex is not maintained outside of an intact cell. Cross-linking procedures may be required to identify interaction and assembly between two proteins.
2. The IgG heavy chain of the immunoprecipitating antibody (that was dispensed in the immunoprecipitation solution) is the same molecular weight as the proteins of interest and masks their presence in Western blots. Resolution: For Western blot detection, use an antibody derived from a different animal host than the immunoprecipitating antibody. In this case, the appropriate secondary-HRP conjugated antibody should not bind appreciably to the immunoprecipitating antibody that was transferred to Western blot membranes from the original immunoprecipitation mixture.